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The Impact of Cigarette / e-Cigarette Vapour on Simulated Pulmonary Surfactant Monolayers under Physiologically Relevant Conditions

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Abstract

Deviation in pulmonary surfactant structure-function activity can impair airway patency and lead to respiratory disorders. This novel study aims to evaluate the influence cigarette / e-cigarette vapour has on model surfactant films located within a simulated pulmonary environment using a lung biosimulator. Chromatographic analysis confirmed that nicotine levels were consistent with the sampling regimen employed. On exposure to smoke vapour, Langmuir isotherms exhibited condensed character and a significant reduction in maximum surface pressure was noted in all cases. Langmuir isocycles, reflective of the human breathing cycle, demonstrated condensed character on smoke vapour delivery. A reduction in the maximum surface pressure was clear only in the case of cigarette vapour application. The components of cigarette vapour can cause oxidative damage to pulmonary surfactant and impair recycling. Neutral nicotine molecules can weaken the structure of the monolayer and cause destabilisation. A protective effect was evident in the case of repeated surfactant compression – relaxation cycles (i.e. the ability to reduce the surface tension term was impaired less), demonstrating a likely innate biological defensive mechanism of the lung. E-cigarette vapour appeared to have a reduced impact on surfactant performance, which may hold value in harm reduction over the longer term.

Key words

Langmuir monolayers, pulmonary surfactant, lung biosimulator, smoking, cigarettes, e-cigarettes, gas chromatography.

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1. Introduction

The primary function of the lung is to permit gaseous exchange between the body and the atmosphere. The main site for such exchange is the alveolar space, which exhibits a moist and highly vascularised surface of approximately 70m² [1]. The naturally occurring fluid that bathes the alveolar lining is subject to considerable surface tension that can force structural collapse on exhalation [2]. In order to counter this effect, and also minimise the work of breathing, a complex and highly surface active mix called pulmonary surfactant is distributed at the alveolar air-liquid interface [3]. The arrangement results in pulmonary surfactant presenting as the initial contacting surface for aerosolised material. Prime examples of such material include respirable therapeutic formulations [4] and, importantly for work presented herein, environmental toxins such as cigarette / e-cigarette vapour [5 & 6].

Pulmonary surfactant is synthesised and secreted by alveolar type II cells located in the deep lung. This endogenous substance exists as an insoluble film that coats the alveolar air-liquid interface [7]. As a result of inherent material characteristics, pulmonary surfactant is capable of reducing the surface tension term to near zero values [8 & 9], which in turn facilitates alveolar stability [3]. In order to achieve this, a dynamic interplay exists between the phospholipid molecules and surfactant specific proteins within the naturally occurring blend. With regard to the former, dipalmitoylphosphatidylcholine (DPPC) predominates and is principally responsible for the surface tension lowering properties of the material [8]. As this amphiphilic molecule undergoes a gel to liquid transition at 41°C, thus the ability to respread across the alveolar air-liquid interface is limited during the breathing cycle [1]. Consequently, additional species are required in order to maintain fluidity and support surfactant respreading. For instance, palmitoyloleoylphosphatidylglycerol (POPG) facilitates effective respreading of pulmonary surfactant following compression [2]. Commercially available lung surfactant replacement preparations (e.g. Survanta®) are frequently prescribed for the management of neonatal respiratory distress syndrome [10]. Such products are often supplemented with palmitic acid (PA), which permits comparable *in vivo* respreading profiles [11]. Thus, throughout this work an appropriate blend of DPPC, POPG and PA is applied to reflect the key lipid fractions of pulmonary surfactant located at the alveolar air-liquid interface.

A Langmuir trough may be used within the laboratory setting to represent the alveolar air-liquid interface [4, 7 & 12]. Here, amphiphilic molecules arrange themselves as per the *in vivo* scenario with their fatty acyl chains displaced away from the supporting aqueous subphase and the polar head groups in direct contact [1]. Scope exists to control environmental parameters with the option to operate at a temperature of 37°C and conduct investigations at elevated relative humidity, as per the (deep) lung; this arrangement may now be investigated via the lung biosimulator [13].

Lateral forces may be applied to simulated pulmonary surfactant monolayers in isolation or indeed succession to achieve expansion / compression cycles reflective of the human breathing pattern [14]. Typical outputs from the approach include Langmuir pressure-area (π -A) isotherms and isocycles, which can be applied to monitor the response of the amphiphilic material when exposed to environmental stressors (i.e. cigarette smoke). For example, in 2003 Bringezu and co-workers applied Langmuir monolayer technology to evaluate the effect of environmental tobacco smoke (ETS) on simulated pulmonary surfactant structure-function activity [11]. The investigation utilised a mixture of DPPC, POPG and PA in the ratio of 69:20:11 to maintain the lipid fraction consistent with clinically used replacement pulmonary surfactant [12]. Here, the surfactant blend was applied to a supporting aqueous subphase that had been previously exposed to ETS. The results from the study suggested that ETS exposure impacts upon monolayer phase behaviour and morphology leading to a higher minimum surface tension (i.e. reduced maximum surface pressure) and impaired lung function.

Tobacco smoking has now become one of the most pervasive habits in modern day society [1]. Tobacco smoke consists of a range of chemical compounds, including aldehydes, amides, amines, carboxylic acids, ketones, esters, phenols and hydrocarbons. The chemical compounds can be further divided into three classes, tobacco-specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs). Compounds assigned to TSNAs, such as N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) comprise of chemicals of known carcinogenic affect, which occur during the manufacturing, fermentation and combustion of tobacco. PAHs, such as naphthalene are located in the particulate composition of tobacco smoke and are produced during the incomplete combustion of the organic material.

104 In order to minimise exposure to the toxic constituents of tobacco smoke, and hence reduce
105 associated long-term deleterious effects, the consumer now has available a range of potential
106 reduced exposure products (PREPs) to purchase [15]. One of the most recently released PREPs is the
107 e-cigarette, which is becoming increasingly popular [16]. As e-cigarettes imitate traditional
108 cigarettes, they not only deliver nicotine but also simulate the process of smoking to satisfy
109 psychological cravings. However, in contrast to traditional cigarettes, e-cigarettes do not involve
110 tobacco combustion. Here, the consumer inhales a vapour that is produced by heating a solution
111 consisting of processed nicotine extract from tobacco leaves, water, glycerine and / or propylene
112 glycol along with flavourings [17]. Potentially harmful constituents present in e-cigarette vapour
113 include carbonyl compounds, volatile organic compounds, TSNA and heavy metals [17]. All can
114 have toxic, irritating and / or carcinogenic effect on the human body [18].

115 This novel study aims to monitor the response of simulated pulmonary surfactant monolayers when
116 challenged with cigarette / e-cigarette vapour under physiologically relevant conditions (i.e. 37°C
117 and elevated relative humidity). For the first time we apply a patented technology platform to
118 quantitatively probe the influence of cigarette / e-cigarette vapour on the performance of a mixed
119 surfactant film located within an environment reflective of the (deep) lung. This work is of interest
120 because it provides a strategy by which to better understand fundamental interactions taking place
121 at a biological interface that is crucial to sustaining life. The timely work will further current
122 understanding of the health impacts associated with smoking cigarettes / e-cigarettes. Throughout
123 the piece consideration will be given to the reproducibility of nicotine presentation within the
124 sampling routine, the identification of chemical species within aerosolised samples and potential
125 mechanisms of interaction with simulated pulmonary surfactant.

2. Materials and Methods

2.1 Materials

The surfactants DPPC (Avanti Polar Lipids, USA. Lot: 160PC-312), POPG (Avanti Polar Lipids, USA. Lot: 160-181PG-131) and PA (Sigma-Aldrich, UK. Lot: PO500) were of analytical grade and used as supplied. Chloroform (CHCl_3) (Sigma-Aldrich, UK) of analytical grade ($\geq 99.9\%$) was employed to clean contacting surfaces and as the spreading solvent. Methanol (HPLC Grade, Sigma-Aldrich, 34860, Lot: STBF7002V) was employed as the solvent during smoke analysis via gas chromatography. Ultrapure water (Purite, UK), demonstrating a resistivity of $18\text{ M}\Omega\text{cm}$, was used both during cleaning procedures and as the Langmuir monolayer aqueous subphase. Marlboro Gold cigarettes along with Blu Classic (first generation) and Eleaf iStick 50W, with Eleaf GS Air Tank atomiser (3rd generation) e-cigarettes were purchased through a retail sources. The strength of the e-cigarette refills was represented by the amount of nicotine (i.e. mg) per 1ml of the liquid solution. The cartridges used with the first generation device contained 18mg of nicotine per unit. The batteries of each device were fully charged before each test to facilitate reproducible data collection.

2.2 Methods

2.2.1 Langmuir Monolayer Preparation

Surfactant monolayers were produced using a Langmuir trough (Model 102M, Nima Technology, UK). Surfactant free tissues (Kimtech Science, Kimberley-Clark Professional, 75512, UK) were soaked in chloroform and used to clean all contacting glassware and surfaces. Background tests to monitor surface pressure in the absence of surfactant material were performed to ensure trough cleanliness, which was accepted at surface pressures of 0.4 mN/m or less on complete barrier compression. A spreading solution composed of DPPC, POPG and PA in the ratio 69:20:11 was produced to reflect appropriate lipid fractions at the alveolar air-lipid interface by dissolving the surfactant material in chloroform to a concentration of 1 mg/ml . In total, $10\mu\text{l}$ of this solution was delivered to the surface of the ultrapure water subphase (50 ml) at pH 7 by dropwise addition using a Hamilton microsyringe. The volume of $10\mu\text{l}$ was chosen so as to achieve a steady transition from the gaseous phase through to condensed phases on barrier compression and prevent saturation of the π -A isotherms / isocycles at the solid phase point.

A period of 10 minutes was allowed to allow chloroform evaporation and surfactant spreading over the 70cm² area. The polytetrafluoroethylene trough barriers were programmed to move to the centre of the trough at a rate of 25cm²/min. Plots of surface pressure vs. percentage trough area for the surfactant system at 37°C and elevated humidity (e.g. 80% RH) were collected using a Wilhelmy plate, formed from Whatman 44 filter paper, at the centre of the compartment.

2.2.2 Cigarette / e-cigarette Vapour Generation

The vapour collection regimen involved taking 2 puffs from the cigarettes / e-cigarettes of 50ml total volume, over a 4-second puff duration with a 30-second puff interval [19]. The vapour was collected in a 250ml quick fit round bottom flask with 3 outlets. Each cigarette / e-cigarette was connected to one of the outlets of the round bottom flask using appropriate tubing. The second outlet, of the same size was connected to a 500ml separating funnel and the third outlet was closed with stopper to produce an airtight system. The experimental arrangement for smoke collection is presented in Figure 1.

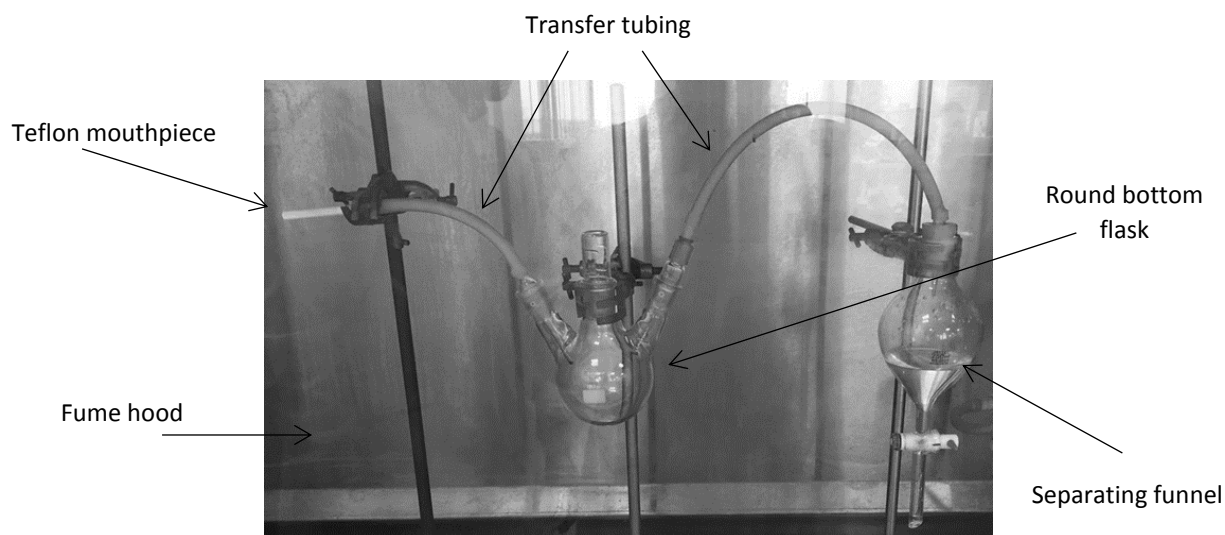


Figure 1. The arrangement applied to collect smoke vapour aliquots.

Before each cigarette / e-cigarette was activated, a total of 100ml of water was poured into the separating funnel (i.e. equivalent to 2 puffs). On activation, smoke vapour was collected in the round bottom flask by withdrawing the 50ml of water from the funnel, with the next puff drawn after 30 seconds [19]. Once the second vapour aliquot was obtained, the round bottom flask containing smoke was disconnected from the separating funnel and mouthpiece and the two outlets are closed with stoppers to hold the smoke inside the flask.

2.2.3 Nicotine Quantification / Smoke Component Determination

Following the collection of each vapour sample, a total of 2ml of methanol was added to the round bottom flask to solubilise the aerosolised material. Each sample was then filtered with a 0.45µm syringe filter into a glass vial insert. Analysis of nicotine standards and smoke extracts was carried out on an Agilent 7980GC with flame ionisation detection (FID). The analytical column selected was an Agilent J&W DB-1 (30m x 0.250mm x 0.50µm), with a column temperature of 160°C (isocratic). The injection type was 1µl split (10:1) (20ml/min 250°C), with nitrogen selected as the carrier gas and the flame ionisation detector temperature programmed at 250°C. Nicotine standards ranging from 0.0078 - 1mg/ml were constructed for nicotine quantification of the vapour extracts. Standards displayed excellent linearity with R² values >0.999. The analysis of 5 replicate smoke samples per cigarette/e cigarette was undertaken.

Evaluation of vapour components was determined using an Agilent 6980GC with 5975MS detection. The column was an Agilent J&W HP5-MSUI (30m x 0.250mm x 0.25µm) with split (10:1) injection of 1µl. The oven temperature were: 50°C for 5mins, 20°C/min to 255°C held for 1 min, 20°C/min to 300°C held for 5 mins. The mass spectrometer was run in full scan mode from 40-500 AMU. Mass spectra for recorded peaks were further evaluated using the NIST database (MS search programme Version 2.0, NIST, MSS Ltd., Manchester, England).

2.2.4 Vapour Addition to Simulated Pulmonary Surfactant Monolayers

In order to assess the impact of smoke vapour on simulated pulmonary surfactant monolayers under physiologically relevant conditions, the aerosolised material was transferred from the round bottomed flask to the enclosed lung biosimulator [13], as detailed in Figure 2, using compressed air. Initially, baseline data was collected in the absence of cigarette / e-cigarette vapour. Subsequently, the smoke vapour acquired from either the cigarettes or e-cigarettes was delivered to the test zone. In each case, a period of 10 minutes was allowed for interaction between each species under consideration.

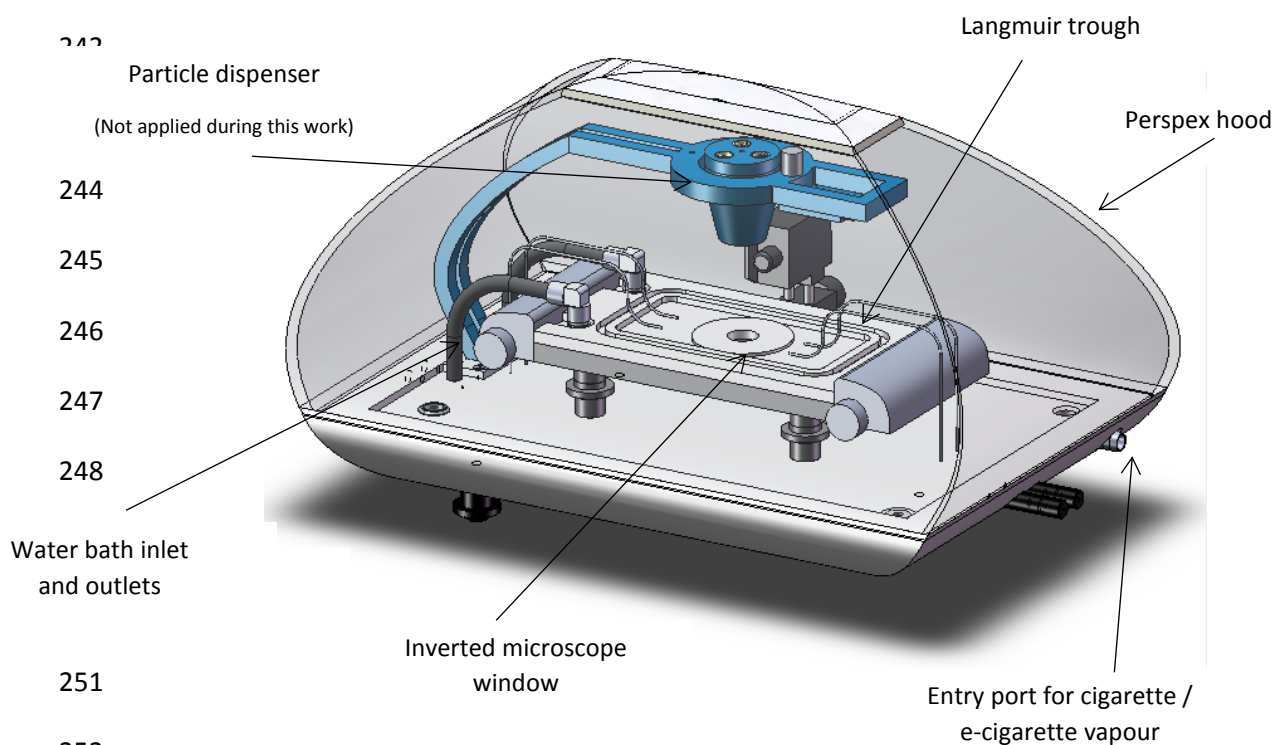


Figure 2. A schematic detailing the lung biosimulator.

To obtain Langmuir isotherms, a single compression was applied towards the centre of the trough at a rate of 25cm²/min. This relatively slow speed was chosen to closely observe the direct impact of cigarette / e-cigarette vapour on both the physical state of the simulated pulmonary surfactant plus compression performance. With respect to Langmuir isocycle tests, a total of 14 compression-expansion cycles were undertaken at a speed of 100cm²/min. This faster compression speed is more representative of the human breathing cycle and provides an insight into system dynamics on exposure to cigarette / e-cigarette vapour. In this case, the first 4 cycles were used to condition the monolayer such that the equilibrium position was attained. This approach enabled a clearer depiction of the influence of the cigarette / e-cigarette vapour on the simulated pulmonary surfactant monolayer. All Langmuir isotherm tests were repeated five times, whilst Langmuir isocycles were repeated three times and averaged data was used to generate the plots presented herein. On test completion, the remaining vapour was removed from the lung biosimulator by directing through a tube to a nearby fume hood using compressed air.

2.2.5 The Compressibility of Langmuir Monolayers

The compressibility term relating to a Langmuir monolayer refers to the ability of the material to lower the surface tension at the air-liquid interface with minimal change in surface area [20]. Surfactant films should ideally have a low compressibility value such that gaseous exchange can take place over a large surface area [21]. The lower the compressibility term, the more rigid the surfactant film is (i.e. the material is of low elasticity), with the opposite being true [22 & 23]. The parameter is calculated as detailed in Equation 1.

$$\text{Compressibility} = \frac{1}{A} \times \frac{1}{m}$$

Equation 1. *Simulated pulmonary surfactant compressibility determination.*

Where A represents the relative surface area and m the slope of the isotherm. Here, 'm' was calculated via $'m' = \frac{y_2 - y_1}{x_2 - x_1}$ over the surface pressure range of 10-30mN/m, whereby 'y' and 'x' values characterise surface pressure and area values, respectively [20].

3. Results & Discussion

3.1 Chemical Analysis of Smoke Vapour and Potential Impact on the Body

Cigarette smoke contains thousands of chemical components, some of which are naturally occurring within the tobacco plant whilst others are added as additives during manufacture [24]. The nicotine component of the Marlboro Gold cigarette vapour tested herein was 0.043mg/ml \pm 0.009, the quantity of this compound corresponded to that stated by the manufacturing company. The 1st generation e-cigarette vapour produced a mean nicotine concentration of 0.048 mg/ml \pm 0.006, with the 3rd generation e-cigarette vapour producing a value of 0.035 mg/ml \pm 0.003. The data demonstrated good reproducibility through all cigarette types.

3.2 Gas Chromatography / Mass Spectroscopy Data

GC-MS analysis of the cigarette / e-cigarette vapour component composition is illustrated in Figure 3a and Figure 3b.

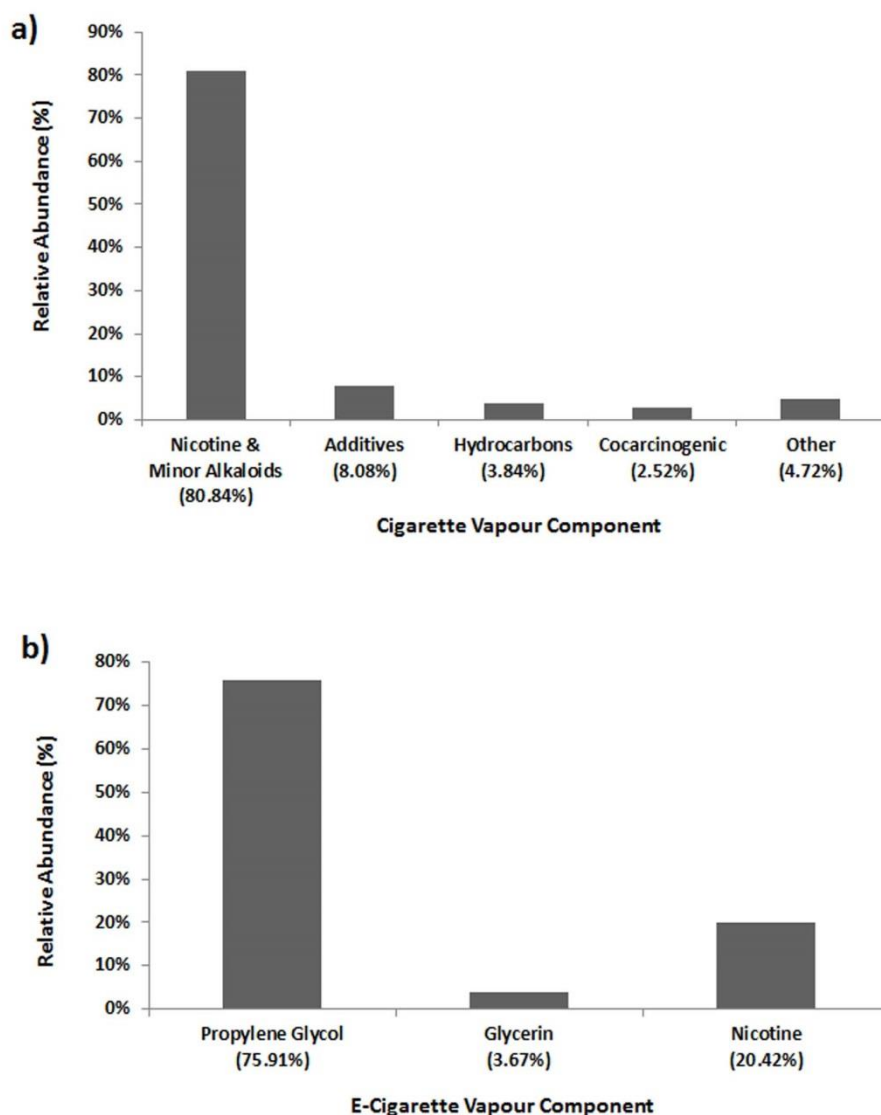


Figure 3. The principal components of cigarette vapour as determined by GC-MS. (a) cigarette vapour; (b) e-cigarette vapour.

The analysis confirms that nicotine and the related minor alkaloid components are the most abundant compounds within the cigarette vapour. In addition, the vapour sample demonstrated a proportion of additive compounds. The compounds representing the 'other' section included amines, and smoke related vapours, such as toluene. With reference to the composition data relating to both the 1st and 3rd generation e- cigarette vapour, it is apparent that nicotine is present, but it is not the major component. The addition of propylene glycol and glycerin to the e-cigarette formulations accounts for a large proportion of the compounds present (i.e. >75% of the total composition) [18].

Toluene and xylene were detected within the cigarette vapour extract by the GC-MS element of this investigation. Exposure to the former can be detrimental to white blood cell function and this can in turn pre-dispose to respiratory tract infections [25]. Furthermore, exposure to xylene at levels greater than 200 ppm can irritate the lungs leading to acute shortness of breath accompanied by chest pain [26].

In terms of the e-cigarette vapour, this route of nicotine administration to the body may be considered less harmful than the more natural, counterpart products. With regard to this system of nicotine delivery, during 2011 Trehy and co-workers documented that the composition of refill products varies considerably as a result it is difficult to fully evaluate the hazards related to electronic cigarette usage [27]. The content of the aerosol generated from e-cigarette is highly variable, not only among different products but also within different samples of the same e-liquids [16, 17, 27, 28, 29 & 30]. Therefore, we suggest that further work is required to better understand the impact of the spectrum of e-cigarette products may have on pulmonary function.

During this work we have carefully replicated the main stages of cigarette / e-cigarette use via reference to a typical puffing regimen [19] and applied the acquired vapour to a test zone housing a model pulmonary surfactant system representative of typical *in vivo* lipid fractions under physiologically relevant conditions [11]. The accepted mechanism of action for pulmonary surfactant, and model mixtures thereof, revolves around the unsaturated lipid fraction (e.g. POPG) forming a fluid-like liquid-expanded matrix to separate phases rich in condensed saturated lipids (e.g. DPPC) [1 & 31]. The delicate coexistence between each phase at the alveolar air-liquid interface is essential for effective surfactant function (i.e. to regulate surface viscosity and lower surface tension) [11, 14 & 31]. Clearly, any disruption to the synergy between the liquid-expanded and liquid-condensed phases forming the surfactant film can have a detrimental impact on gross lung function [1 & 21]. Within the laboratory setting, deviation in recorded Langmuir pressure-area isotherms and / or isocycles provides direct evidence of changes to overall surfactant performance.

3.3 Langmuir Pressure – Area Isotherms

Langmuir pressure-area isotherms were acquired for the simulated pulmonary surfactant systems when exposed to either cigarette or e-cigarette vapour under conditions reflective of the (deep) lung; relevant data are presented in Figures 4 and 5, respectively. All systems exhibit two-dimensional phase changes over the course of compression; movement through the gaseous, expanded and condensed phases is confirmed on gradient change from right to left. Here, the compressibility parameter was considered with the slope of the trace used as a marker for the compressibility of the two-dimensional film; where the steeper the slope, the harder it is to compress the surfactant monolayer [32].

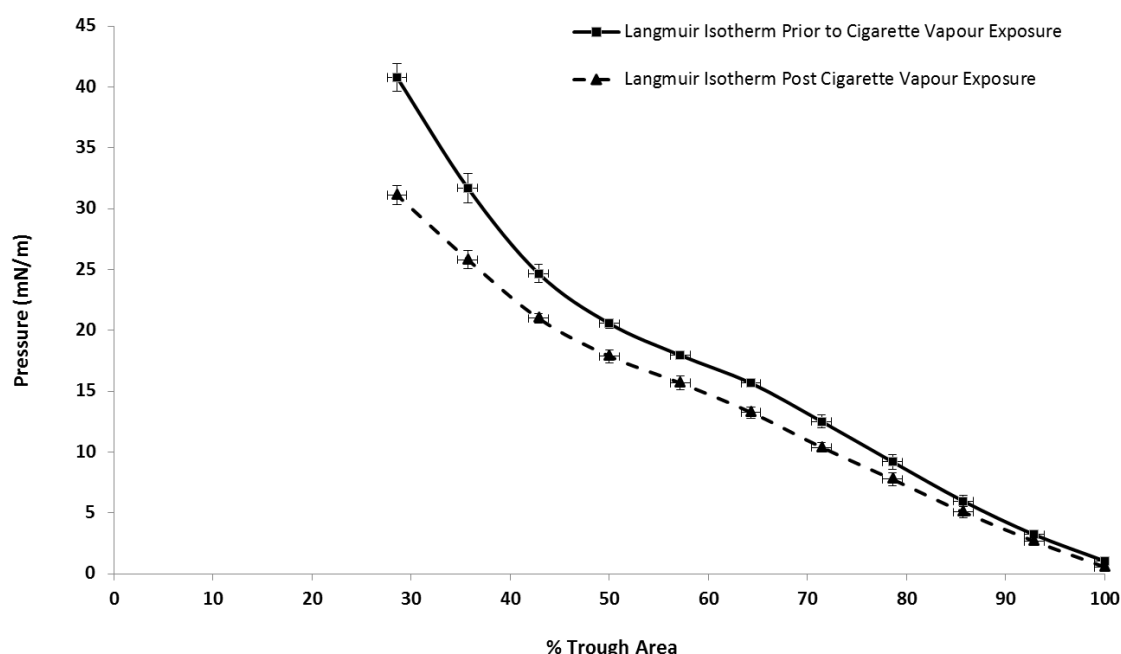


Figure 4. A Langmuir pressure-area isotherm detailing the response of a simulated pulmonary surfactant monolayer to cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated relative humidity. Averaged data of 5 replicates presented with standard error of the mean displayed.

On inspection of the data presented in Figure 4, it is clear that the administration of cigarette vapour to the test zone did influence simulated pulmonary surfactant structure-function activity. Here, the ability to attain low surface tension values at any given relative area is reduced and there is an increase in the ease of compression under physiologically relevant conditions (i.e. the monolayer is more compressible).

In the case of the model surfactant system studied herein, the highest surface pressure recorded in the absence of cigarette smoke was 41mN/m. This value was as a direct result of applying 10µl of the surfactant spreading solution (1mg/ml) to the supporting aqueous subphase, which was deemed appropriate to achieve smooth lipid phase transitions during compression and prevent solid phase saturation at minimal trough areas. If a larger spreading solution volume were to be applied to the aqueous subphase then the maximum surface pressure would rise (e.g. attain a value of approximately 70mN/m). On application of cigarette vapour, the value of 41mN/m diminished to 32mN/m. Hence, the capacity to lower the surface tension at full monolayer compression was reduced by 22%. In addition, exposure of cigarette vapour resulted in the monolayer exhibiting a condensed character (i.e. being transposed to the left of the baseline plot). Comparable trends, as those noted here, would be anticipated at higher surface pressure values (e.g. 70mN/m) [11].

A similar response was noted when 1st and 3rd generation e-cigarette vapour was delivered to the test zone. Once again the baseline plot for our system exhibited a maximum surface pressure of 41mN/m (i.e. due to the application of 10µl of material) with reduction in the term evident on exposure to 1st generation and 3rd generation e-cigarette vapour; namely 32mN/m and 36mN/m, respectively. It is interesting to note that on delivery of the 1st generation e-cigarette vapour an identical reduction in the surface pressure term of 22% was noted. This deviation was less in the case of the 3rd generation product, namely a 12% reduction. The presence of e-cigarette vapour led to a reduction in the maximum surface pressure from the baseline data, this finding is statistically significant due to the absence of overlap in the presented standard error of the mean bars. Furthermore, as previously noted exposure to e-cigarette vapour caused a clear decrease in surface pressure at any corresponding area.

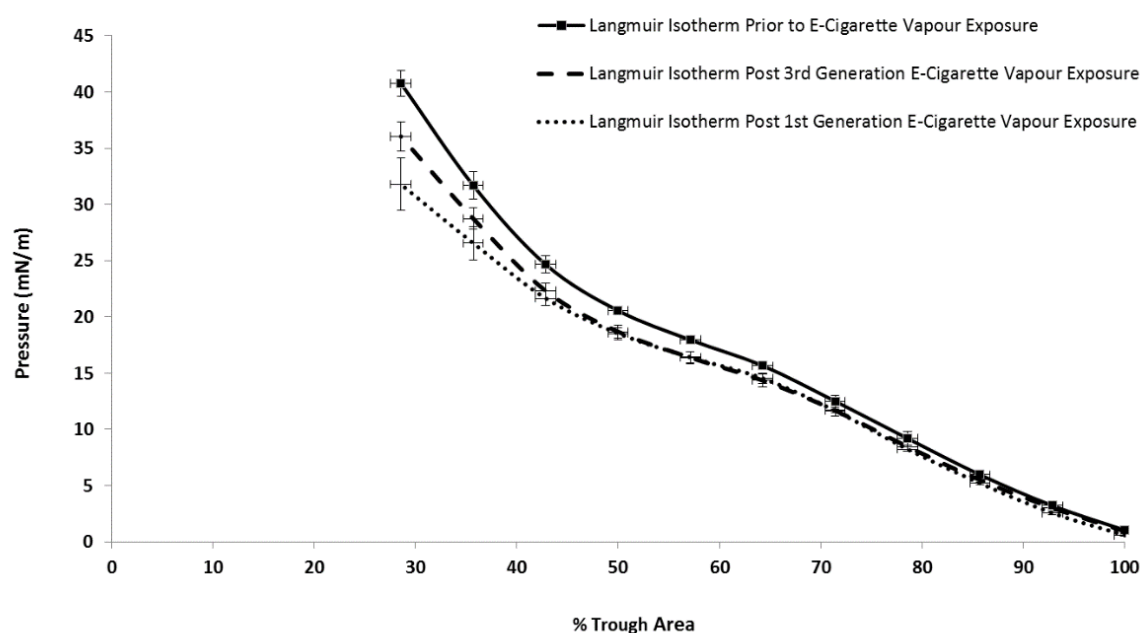


Figure 5. *Langmuir pressure-area isotherm data outlining the response of a simulated pulmonary surfactant monolayer to e-cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated relative humidity. Averaged data of 5 replicates presented with standard error of the mean displayed.*

Similar responses to those outlined above have been noted within the literature [11]. All data presented within this piece are reflected of the *in vivo* situation where smoke vapour would interact with pulmonary surfactant via a ‘top-down’ approach. In this instance, the hydrocarbon chains of the phospholipid molecules were primarily exposed to those chemicals within the smoke aliquots. Therefore, this work considers real-world interfacial interactions that can potentially compromise the biological function of the lung. Furthermore, in support of our findings Kannisto and Yhteiskoulu reported functional changes in the lipid fraction of pulmonary surfactant as a result of phospholipid degradation and / or the penetration of nicotine molecules into the two-dimensional film during their 2006 study [33].

3.3.1 Langmuir Isotherm Compressibility Analysis

In order to quantify the impact of cigarette / e-cigarette vapour had on simulated pulmonary surfactant compressibility Equation 1 was applied. Here, the slope of the Langmuir pressure-area isotherm was considered along the liquid-expanded to liquid-condensed transition. That is to say between the surface pressures of 10mN/m to 30mN/m at the specific relative trough areas of 40%, 50% and 70%. Compressibility data for each system is presented in Figure 6.

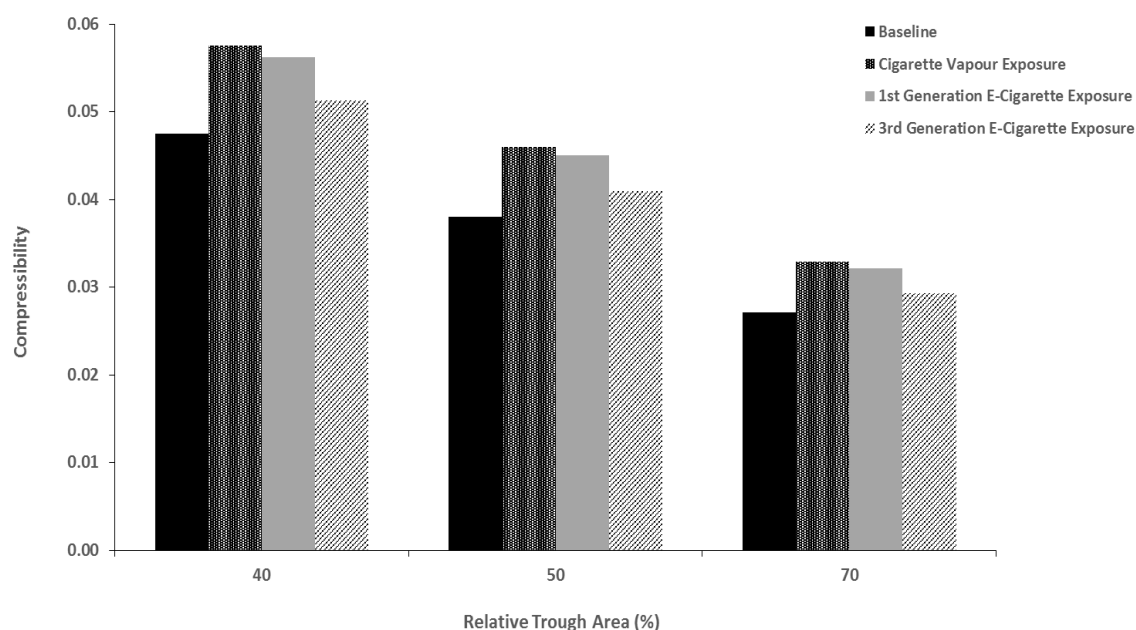


Figure 6. The compressibility of simulated pulmonary surfactant monolayers at pre-defined relative trough areas in the absence and presence of cigarette / e-cigarette vapour. In all cases of single monolayer compression (i.e. Langmuir isotherms), the delivery of such vapour to the test zone increased the compressibility term.

On exposure to cigarette / e-cigarette vapour, the compressibility term increased in all cases. Greater compressibility values indicate that the surfactant film becomes less rigid in nature and more elastic (i.e. easier to compress when compared to the baseline). This effect is more pronounced in the case of exposure to cigarette vapour. The impact on monolayer compressibility is limited in the case of the 3rd generation e-cigarette.

Although the use of Langmuir isotherms is not representative of the human breathing cycle, which is dynamic in nature, we believe that the information obtained from this largely static system can provide insight into the way in which environmental toxins (e.g. cigarette / e-cigarette vapour) can influence individual molecular species that are in the main fully exposed at the alveolar air-liquid interface (i.e. when in the gaseous phase). Here, we liken this situation to a lone soldier under attack from an opposing force.

In all cases, exposure to cigarette / e-cigarette vapour resulted in the simulated pulmonary surfactant monolayer exhibiting a condensed character. Consequently, the ability to reduce the surface tension term was impaired across all relative trough areas during compression to the centre of the compartment. In addition, there was an apparent increase in monolayer compressibility. Clearly, exposure to vapour from all platforms had a detrimental impact on simulated pulmonary surfactant performance with exposure to cigarette vapour and the 1st generation e-cigarette vapour being the most significant. There are a number of reasons to explain the notable trend in the data sets presented herein. A previously reported aspect involves a reduction in phospholipid content within the surfactant film due to exposure to the chemical constituents of smoke vapour (e.g. free radicals and oxidising agents) [11]. Importantly, we believe that a key mechanism of surfactant film degradation lies in the ability of neutral nicotine molecules within smoke vapour to penetrate in-between the relatively exposed phospholipid polar head groups of the surfactant film. On inhalation, nicotine in the unionised form is able to enter the body and can readily pass across membrane structures as opposed to protonated nicotine [34]. As such, the tobacco industry typically designs cigarettes to have a large proportion of unprotonated nicotine for inhalation to enhance lung deposition and delivery to the brain [35]. Consequently, when the surfactant film is in the uncompressed state (i.e. with the individual surfactant molecules decidedly exposed for interaction) neutral nicotine could potentially weaken intermolecular van der Waals forces and cause structural destabilisation, which will ultimately increase the compressibility of the material (i.e. cause it to be less rigid) [33].

Tobacco-specific nitrosamines can also have a detrimental impact on the mechanical properties of surfactant monolayers (i.e. by degrading individual phospholipid molecules) [36]. For example, NNN and NNK are primary carcinogenic tobacco-specific nitrosamines that are present in cigarette smoke [37]. Upon interaction with a surfactant film, these agents enhance phospholipid hydrolysis and subsequently reduce content within the alveolar space; an accompanied increase in lysophospholipid is also noted [28]. Within the body, lysophospholipids are formed as a result of phospholipase A2 stereoselective hydrolysis of the ester linkage of phospholipids to release fatty acids and lysophospholipids [38]. The lysophospholipids produced also have a direct detergent-like effect on the surfactant leading to impaired surface activity and consequently lead to a reduction in rigidity across the two-dimensional plane [21].

3.4 Langmuir Pressure – Area Isocycles

Langmuir pressure-area isocycles were also recorded for each system under conditions reflective of the *in vivo* scenario such that the impact of smoke vapour on surfactant dynamics could be assessed; representative plots are presented in Figures 7, 8 and 9. Again, the presence of cigarette / e-cigarette vapour within the test zone did impact simulated pulmonary surfactant function. In each case, the surfactant film exhibits a condensed character and the ability to lower the surface tension at all stages throughout compression is weakened.

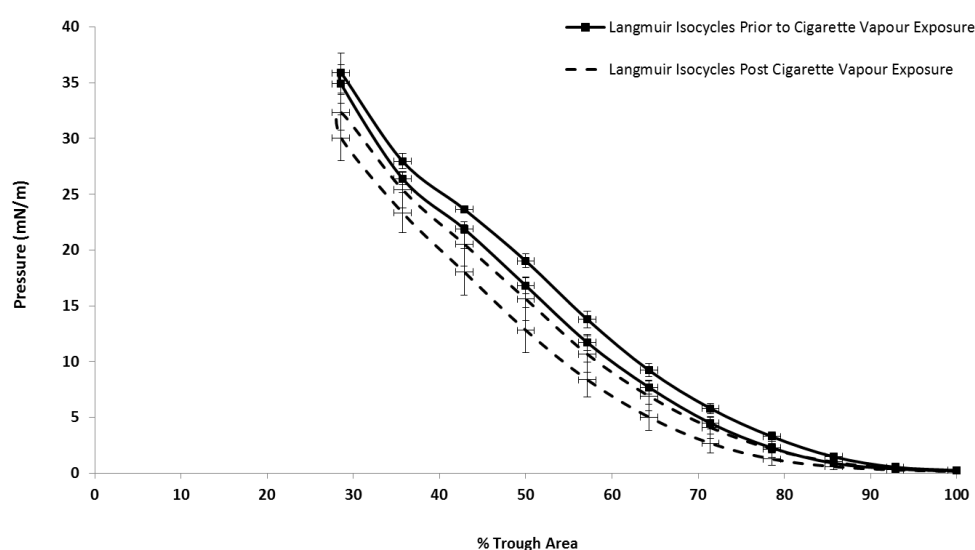


Figure 7. Langmuir pressure-area isocycle data relating to the response of a simulated pulmonary surfactant monolayer to cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated relative humidity. Averaged data of 3 replicates presented with standard error of the mean displayed. Where, each replicate consists of 10 compression-expansion cycles at a barrier speed of 100cm² / min.

With regard to the baseline systems (i.e. Langmuir isocycles in the absence of cigarette / e-cigarette vapour), the maximum recorded surface pressure was 36mN/m during this work on addition of 10μl spreading solution to the surface of the supporting aqueous subphase. This value is comparable to that previously observed for the Langmuir isotherm element of this study, with the slight reduction due to monolayer pre-conditioning (i.e. the execution of 4 compression – expansion cycles) to attain the equilibrium state.

Following exposure to cigarette vapour, the ability of the simulated pulmonary surfactant film to reduce the surface tension term was impaired at all relative trough areas. The result may be ascribed to a reduction in the total phospholipid / lipid content of the surfactant film [14 & 21]. Moreover, if the gradient of the trace between the surface pressures of 10mN/m and 30mN/m is considered, it is apparent that the surfactant film exposed to the cigarette vapour is less compressible (i.e. harder to compress) when compared to the baseline isotherm. Thus, the data indicate that exposure to cigarette vapour increases the work required to compress the simulated pulmonary surfactant monolayer to the minimum trough area.

On expansion, the simulated pulmonary surfactant monolayer exposed to cigarette smoke followed a similar pattern to that of the baseline system. The result confirms that the material is able to respread after exposure to smoke vapour. Furthermore, the apparent hysteresis between compression and expansion cycles was constant. Interestingly, the difference in collapse pressure before and after exposure to smoke was less significant compared to the single compression isotherm presented in Figure 4; in this case only an 11% reduction was calculated for the term. We attribute this result to a 'protective mechanism' on dynamic monolayer compression – expansion cycling and suggest that the lipid peroxidation effects contribute to the chemical degradation of the POPG molecule that is primarily responsible for maintaining the fluidity of the surfactant film.

Following exposure to e-cigarette vapour, the simulated pulmonary surfactant monolayers were not significantly degraded and once again displayed condensed character as illustrated in Figures 8 and 9. Here, the ability to lower the surface tension term at all relative areas was reduced, as previously noted in the case of the cigarette vapour addition. In contrast to the previous system, the data confirm that the maximum surface pressure of 36mN/m is attained subsequent to e-cigarette vapour exposure. Thus, there is limited impact on attaining the maximum surface pressure value.

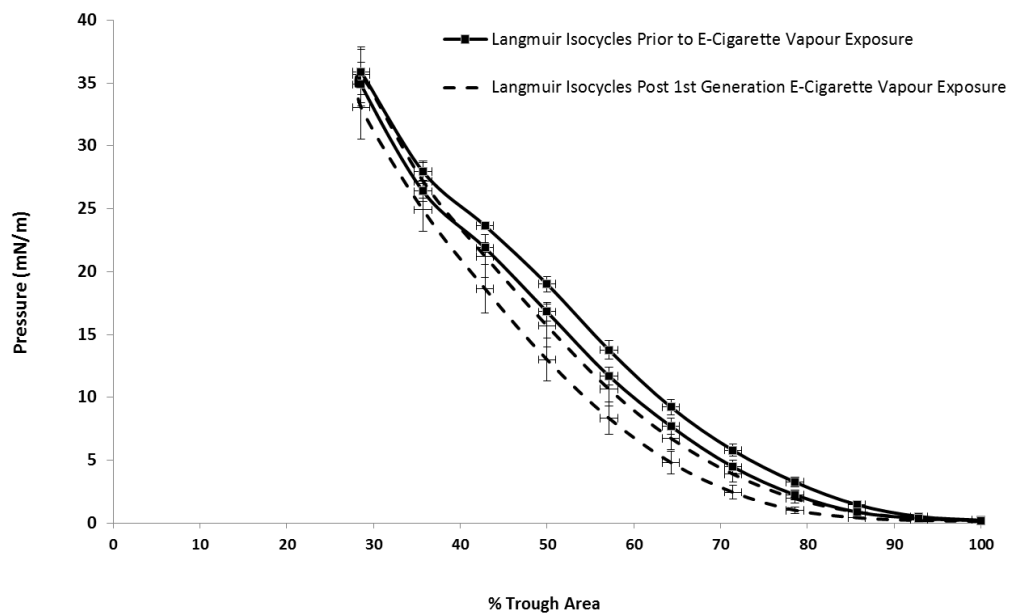


Figure 8. Langmuir pressure-area isocycle data relating to the response of a simulated pulmonary surfactant monolayer to 1st generation e-cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated relative humidity. Averaged data of 3 replicates presented with standard error of the mean displayed. Where, each replicate consists of 10 compression-expansion cycles at a barrier speed of 100cm² / min.

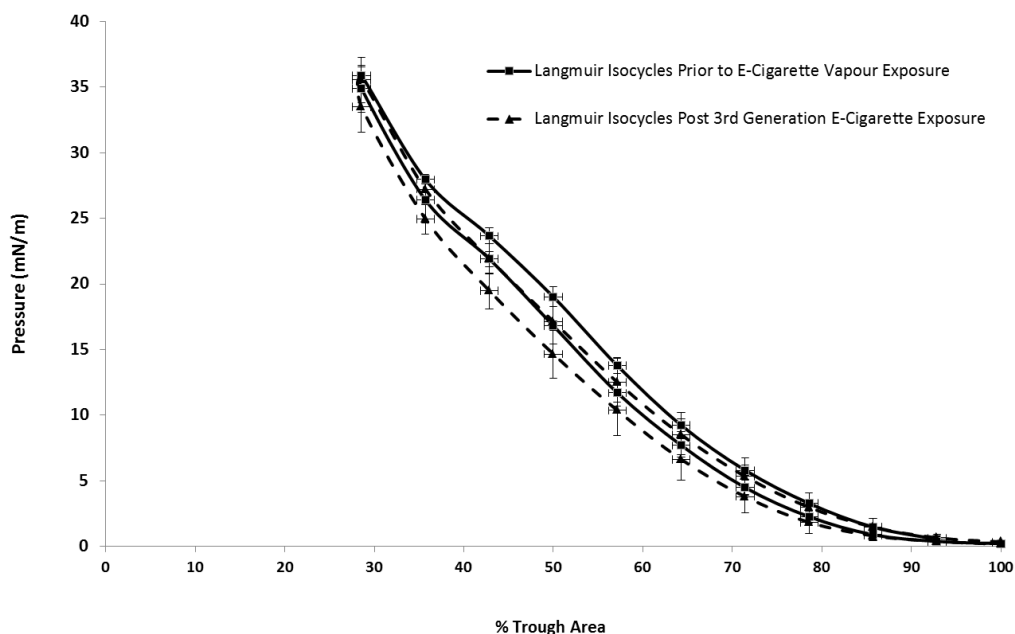


Figure 9. Langmuir pressure-area isocycle data relating to the response of a simulated pulmonary surfactant monolayer to 3rd generation e-cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated relative humidity. Averaged data of 3 replicates presented with standard error of the mean displayed.

We attribute the apparent deviation between each Langmuir isocycle to both the loss / degradation of amphiphilic material at the air-liquid interface and the penetration of nicotine molecules between the polar head groups of the constituent molecules [11 & 33]. The reduction in the surface pressure is more pronounced upon exposure to the vapour generated from the 1st generation e-cigarette. Here, there is a clear translocation to the left within the plot when compared with baseline starting from approximately 1mN/m up towards 28mN/m. Such deviation is not as apparent shift in the case of exposure to 3rd generation e-cigarette vapour. In the case of exposure to both 1st and 3rd generation e-cigarette vapour exposure, the hysteresis between the expansion and compression phases are of similar sizes to that presented within the baseline.

3.4.1 Langmuir Isocycle Compressibility Analysis

In a similar fashion to that previously described, consideration was given to the quantitative determination of the influence cigarette / e-cigarette vapour had on simulated pulmonary surfactant compressibility during active cycling; once again Equation 1 was applied. Here, the slope of the Langmuir pressure-area isocycle was considered along the liquid-expanded to liquid-condensed transition. That is to say, between the surface pressures of 10mN/m to 30mN/m at the specific relative trough areas of 40%, 50% and 70%. Compressibility data for each system is presented in Figure 10.

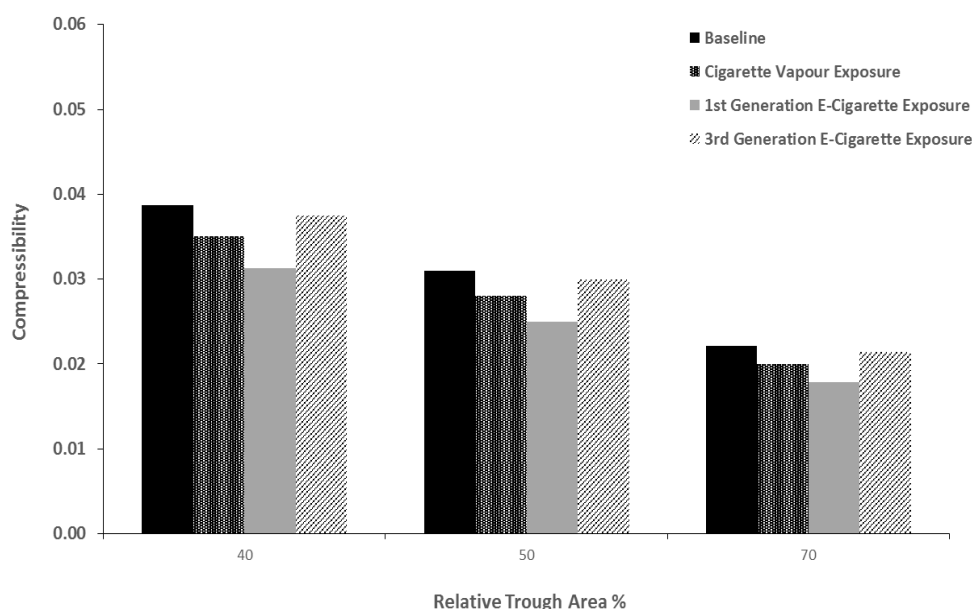


Figure 10. The compressibility of simulated pulmonary surfactant monolayers at pre-defined relative trough areas in the absence and presence of cigarette / e-cigarette vapour. In all cases of repeated monolayer compression-expansion (i.e. Langmuir isocycles), the delivery of such vapour to the test zone decreased the compressibility term.

Following exposure to cigarette / e-cigarette vapour, the compressibility term decreased. Lower compressibility values indicate that the surfactant film became more rigid in character and thus harder to compress when compared to the baseline. This effect was more pronounced in the case of the 1st generation e-cigarette vapour, demonstrating a potentially greater adverse effect on pulmonary surfactant activity. As per previously noted, the influence on monolayer compressibility is minimal in the case of the 3rd generation e-cigarette; this point supports the usefulness of the more recently developed electronic products (e.g. PREPs) to support harm reduction within the population.

The use of Langmuir isocycles closely represents the *in vivo* scenario. In this case, the collection of amphiphilic molecules experience a two-dimensional lateral force on trough barrier movement to the centre of the compartment with the phospholipid head groups less accessible to environmental toxins and hence may be described as 'protected'. During surfactant compression-expansion cycles, the fluid phase associated with surface active material is rapidly exchanged between the monolayer interface and the adjoining surface associated reservoir [14 & 31]. As the monolayer is compressed, the increase in surface pressure directs a fraction of the unsaturated lipid component (i.e. POPG) away from the interfacial zone to desorb into the surface-associated, multilayer reservoir [39]. On expansion, these fluid phase components stored in the surface associated reservoir support the readsorption of the lipid fraction back to the interfacial zone [31]. The presence of cigarette / e-cigarette vapour within the vicinity of a surfactant film inhibits such exchange mechanisms and therefore alters the proportion of phospholipids within the two-dimensional monolayer [14]. As such, the mechanical properties of the monolayer film are adversely affected (i.e. there is an apparent increase in film rigidity) which ultimately impairs the surface tension lowering capacity of the material [11].

This point is confirmed by the apparent decrease in monolayer compressibility and impairment in the ability to reduce the surface tension term at all relative trough areas. A number of mechanisms have been proposed to explain such findings and include for example the presence of oxygen derived free radicals within cigarette vapour that are capable of reducing the amount of unsaturated lipids (i.e. POPG) within the two-dimensional ensemble via peroxidation of double carbon-carbon bonds within the acyl chains [40]. The net result is the presentation of a rigid interface that is high in solid phase domains. This type of reaction involves the oxidative degradation of the amphiphilic species by free radicals contained within cigarette vapour [41].

The oxidation of unsaturated components within a lipid monolayer (i.e. the exposed acyl chain groups of the ensemble) is anticipated due to the availability of multiple double bonds accompanied by methylene bridges that possess especially reactive hydrogen atoms [42]. Naturally, a reduction in the liquid phase within a rigid monolayer leads to poor respread profile on expansion and reduced surfactant coverage at the air-liquid interface [43].

The data presented within this study clearly demonstrate that exposure to cigarette / e-cigarette vapour has a detrimental impact on the activity of a simulated pulmonary surfactant film. The amphiphilic material forming the surfactant monolayer is central to the regulation of the surface tension parameter at the alveolar air-liquid interface [14 & 21]. As such, if we take the findings presented within this study and extrapolate to the *in vivo* scenario, an increase in the work of breathing would be anticipated. The net effect of this would be impaired lung function, which could manifest as compromised gaseous exchange within the (deep) lung, potential collapse or incomplete inflation of the lung structure itself, hypoxia, oedema and quite possibly pulmonary hypertension [41 & 44]. Furthermore, due to such deviation from the healthy state, scope exists for longstanding conditions to develop including for example chronic obstructive pulmonary disease (COPD) along with interstitial lung disease. Overall, impairment to lung mechanics would be expected [44]. Indeed, previous work has confirmed significant reductions in phospholipid concentrations in the bronchoalveolar lavage fluid obtained from those who smoke cigarettes and experience COPD [45 & 46]. Thus, the lung-specific adverse effects associated with cigarette smoking can reduce the quality of life of the individual and increase the likelihood of premature death.

Over the course of recent years, e-cigarettes have become increasingly popular within developed countries because of the possibility of delivering nicotine to the body in a clean format whilst concurrently satisfying behavioural triggers [17, 29 & 47]. In relation to this point, during 2014 Safari and co-workers documented the fact that e-cigarettes can reliably deliver nicotine to the lung whilst limiting the exposure to tobacco specific toxins when compared with traditional cigarettes and the use of hence it is a healthier alternative from a public health perspective [48]. However, potential drawbacks to the wide spread uptake of e-cigarettes involve the lack of quality control and manufacturing regulations currently in place. For instance, such regulations do not fully cover aspects comprising raw material inclusion, purification stages and batch-to-batch consistency of e-liquid refills; all of which can impact upon the vapour profile from the respective products [17, 18, 48 & 49]. Clearly, these elements require further detailed investigation.

Although not reported here, some commercially available e-liquid and cartridge refills do contain chemicals that may pose potential health risks to the individual; interestingly these agents have also been detected within tobacco smoke vapour [16, 17, 18, 27, 47 & 48]. For example, the cytotoxic and carcinogenic substances including formaldehyde, NNN, NNK and acrolein have been identified within e-cigarette vapour; all may have deleterious effects on the human body [16, 17 & 48]. Although the concentration of such substances is much lower than in traditional cigarette vapour, alteration of pulmonary surfactant activity is possible at the alveolar air-liquid interface and this can in turn initiate the presentation and development of the lung related complications / disease states listed above [1, 11 & 50].

4. Conclusion

This study has demonstrated that exposure to cigarette / e-cigarette vapour does modify the structure-function activity of simulated pulmonary surfactant monolayers under physiologically relevant conditions. The results offer insight into the potential effects such (environmental) toxins can have on the human lung. With reference to the dynamic system investigated herein, the capacity to reduce the surface tension term was impaired throughout and the compressibility of the surfactant film was reduced in all cases. The findings were ascribed to the chemical interactions taking place between pulmonary surfactant-specific components and the smoke vapour delivered to the test zone. We propose key mechanisms of interaction include: a) nicotine insertion into the two-dimensional phospholipid ensemble, b) lipid peroxidation of the amphiphilic acyl chains and c) hydrolysis of the phospholipid chains via tobacco-specific nitrosamine association.

Detrimental interactions such as these can cause molecular destabilisation and inhibit phospholipid exchange with the surface associated reservoir system. Correspondingly, a reduction in lung compliance can lead to the development of a range of lung specific complications including pulmonary oedema and COPD; the latter condition is frequently noted with the chronic smoker. Undoubtedly, further work is required to gain greater insight into the delicate interplay between environmental toxins and the pulmonary space. Such investigation may now be readily conducted via use of the lung biosimulator platform presented within this piece. Here, scope exists to consider the influence of a wide range of environmental toxins have on lung function, including for example petrol and diesel fumes. This device also holds potential to quantitatively probe the interaction between respirable therapeutic formulations and the deep lung (e.g. in pharmaceutical dissolution testing).

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